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Tissue culture of human kidney epithelial cells of proximal tubule origin

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Tissue culture of human kidney epithelial cells of proximal tubule origin. The in vitro culture of human kidney epithelial cells of defined nephronal origin would prove valuable in a variety of studies defining the factors and mechanisms responsible for diseases and disorders of the kidney. In this study we have tested the hypothesis that employing a serum-free growth medium allows the selective cultivation of human kidney epithelial cells. It is demonstrated that human kidney cortex, explanted into serum-free hormonally defined growth medium gives rise to a primary culture of kidney epithelial cells of homogenous morphology capable of hemicyst formation. While these cells were able to proliferate to confluency as an explant culture, they were unable to undergo stable subculture. Subsequent manipulation of the culture vessel surface (an initial coat of bovine type I collagen followed by the absorption of macromolecules from fetal calf serum) yielded cultures able to be subcultured with growth to at least 30 cell generations. These cells were identified to be of proximal tubule origin by employment of enzyme histochemistry, immunohistochemistry, and ultrastructural examination.

Culture tissulaire de cellules épithéliales de reins humains d'origine tubulaire proximale. La culture in vitro de cellules épithéliales de reins humains d'origine néphronique bien définie pourrait s'avérer utile dans de nombreuses études définissant les facteurs et les mécanismes responsables des maladies et de règlements du rein. Dans cette étude, nous avons testé l'hypothèse selon laquelle l'emploi d'un milieu de croissance sans sérum permet la culture sélective de cellules épithéliales de rein humain. Il est démontré que le cortex rénal humain explanté dans un milieu de croissance sans sérum, bien défini au point de vue hormonal, donne naissance à une culture primaire de cellules épithéliales rénales de morphologie homogène, capable de former des hémicystes. Bien que ces cellules soient capables de proliférer jusqu'à confluence en culture à partir d'explants, elles sont incapables de subir une culture secondaire stable. Une manipulation ultérieure de la surface du récipient de culture (une couche initiale de collagène bovin de type I suivi par l'absorption de macromolécules provenant de sérum de veau foetal) a permis des cultures capables d'être cultivées de façon secondaire avec une croissance d'au moins 30 générations cellulaires. Ces cellules ont été identifiées comme étant d'origine tubulaire proximale, en utilisant un examen enzymatique histochimique, immunohistochimique, et ultrastructural.

Human kidney epithelial cells of defined origin, able to be propagated in vitro, would prove valuable for studies defining the pathological and biochemical alterations of renal cell functions in diseases and disorders of the kidney. The employment of in vivo studies has as a major obstacle the cellular diversity within the nephron itself and the fact that most of the pathological and biochemical alterations in disease states are unique for a particular nephron segment or even for a specific glomerular or tubular cell type. In addition, in vivo studies aimed at isolating a specific nephron segment further suffers from a lack of sufficient final cell quantity for detailed study. An approach to overcome these innate limitations would be to prepare homogeneous differentiated renal cell populations of defined nephronal origin able to be serially propagated in vitro. This would yield sufficient cell mass in which both acute and chronic studies on normal and altered cell function could be performed.

Essentially three approaches to cell culture methodology were used to provide kidney cells. The first approach involved using existing animal cell lines of kidney origin [1–5]. The major deficiencies of this approach were that the exact site of origin within the nephron is unknown, and that being a cell line, it may not be totally representative of the normal state and may in fact possess tumorigenic properties. The second approach was the isolation of specific nephron segments by dissection and sieving. Primary growth established by these explant techniques or protease digestion was followed by cloning to assure uniform cell type [6, 7]. This type of approach greatly facilitates defining the site of origin within the nephron but has as a limitation the number of cells which can be grown for study since, when cloned, many generations of potential cell divisions are expended [8]. The third and newest approach was the digestion or explantation of kidney tissue in the presence of serum-free, hormonally defined culture media such that fibroblast proliferation was minimal or eliminated and epithelial cell growth encouraged [9–11]. The advantage of this type of approach is the possibility that depending on media composition only a defined type of epithelial cell may be able to proliferate. This would further the possibility that defined segments of the nephron could be cultured simply by adjusting media composition and the problems associated with cloning and tedious microscopic dissection of specific kidney elements would be eliminated. In this work we report the tests of the ability of a serum-free hormonally defined culture medium to allow the explant and subsequent subculture of human kidney epithelial cells of proximal tubule origin.

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Methods

Reagents

Dulbecco's Modified Eagles' Medium (DME) and Ham's F-12 (F-12) growth medium were obtained from Gibco Diagnostics, Grand Island, New York. Fetal calf serum (fcs) and trypsin-versene solution (0.05%, 0.02%) were obtained from Biofluids, Rockville, Maryland. Serum-free growth media components were obtained from Collaborative Research, Lexington, Massachusetts. Bovine type I collagen was obtained from Flow Laboratories, McLean, Virginia. Reagents for the preparation of routine solutions were the highest quality available from Fisher Scientific, Atlanta, Georgia. Enzymes and coenzymes for histochemistry were obtained from Sigma Chemical Company, St. Louis, Missouri. Tissue culture plasticware was of the Corning trademark and obtained from Fisher Scientific. Triple-distilled water was utilized for the preparation of cell culture reagents. Antiserum to human factor VIII RAg and Tamm-Horsfall glycoprotein were obtained from Cappel Laboratories, Cochranville, Pennsylvania. Antiserum to keratin was a gift from Dr. Howard Green, Harvard Medical School, Boston, Massachusetts. Antiserum to human carbonic anhydrase was a gift from Dr. R. E. Tashian, University of Michigan Medical School, Ann Arbor, Michigan.

Kidneys for explant

Human kidneys utilized for establishing explant cultures were obtained from three sources. Cadaver kidneys donated for transplant which did not meet specifications were obtained after accession and examination in the Surgical Pathology Department of the Medical University of South Carolina, Charleston, South Carolina. Kidneys were also obtained from autopsy cases initiated within 6 hr of death from subjects unaffected with kidney or kidney-related disease. Portions of kidney cortex uninvolved in the disease state were also obtained from kidneys removed at surgery for such diseases as renal cell carcinoma. In these cases tissue was obtained following accession and examination by Surgical Pathology and after completion of all diagnostic protocols. The use of human tissue was reviewed and approved by the Medical University of South Carolina Institutional Review Board for Human Research.

Kidney explants

When the kidney arrived at the cell culture facility, it was placed in a laminar flow hood and longitudinally sectioned using sterile techniques. The fibrous capsule was removed and approximately 1-cm^3 portions of tissue removed from the outer cortex were placed in a 100-mm petri dish containing DME culture medium. The tissue sample was then cut with scissors and forceps into pieces approximately 1 mm^3. Six tissue fragments were placed on the growth surface in each 25 cm^2 T-flask which had been previously coated with collagen. The flasks were placed in an inverted position to facilitate fragment attachment for 30 min at room temperature. The flasks were then righted and 2.5 ml of a 1:1 mixture of DME and F-12 growth medium containing insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and epidermal growth factor (10 ng/ml) were added. The explants were placed at 37°C in a 5% CO_2:95% air incubator and left undisturbed for 3 days. The cultures were fed fresh growth medium every 3 days until confluent.

The preparation of matrix substrates for coating the growth surface of culture vessels were prepared and utilized following the manufacturer's instructions. Bovine collagen type I was obtained from Flow Laboratories. Human fibronectin was obtained from Collaborative Research. For the preparation of a collagen and fibronectin matrix the flask was first treated with a collagen solution (1.3 mg/ml); the excess collagen was removed and the surface was allowed to air dry. The flask was then rinsed three times with PBS, and a solution of fibronectin (5 μg/cm^2 of surface area) was added for 45 min at room temperature. The fibronectin solution was then removed and the growth surface was rinsed three times with PBS. For the preparation of a collagen/fetal calf serum matrix, the growth surface was first treated with collagen as described above. Then, fetal calf serum was added (0.2 ml/cm^2 of growth surface) and allowed to incubate for 12 hr at 4°C. The fcs was then removed and the growth surface was rinsed five times with PBS.

Subculture of cells

When confluent, the cell monolayers were subcultured by rinsing the monolayers twice with phosphate-buffered saline (PBS) followed by the addition of trypsin-EDTA (0.05%, 0.02% in Hank's balanced salt solution). Cell detachment was monitored by light microscopy and in no instance was treatment allowed to proceed for longer than 4 min. Cell detachment was further facilitated by striking the flask against the palm of the hand. Trypsin action was halted by the addition of an equal volume of fcs or 0.1% soybean trypsin inhibitor. The detached cells were transferred to a 15-ml centrifuge tube, brought to volume with PBS, and centrifuged at $\times 800g$ for 5 min. The cell pellet was recentrifuged in PBS, resuspended in culture media, and distributed to new flasks at a 1:3 subculture ratio. For the determination of cell doubling times, the cells were subcultured into a series of triplicate 25 cm^2 T-flasks at a 1:3 subculture ratio. The cells were then removed from the culture surface at 24-, 48-, 72-, 96-, and 120-hr intervals. The number of cells were determined utilizing a Model B Coulter Counter. For cryopreservation of cells, the cell pellet was resuspended in serum-free growth medium containing 10% DMSO. The vials were placed inside a styrofoam container in a −70°C freezer for 1 hr and then transferred for storage to a Dewar flask used for liquid nitrogen. For recovery, the vials were thawed at 37°C and placed into fresh serum-free medium.

Photography

Photomicrographs were recorded on a 35 mm Kodak Panatomic X film, ASA 32, with the use of an Olympus 1 M inverted microscope.

Electron microscopy

Monolayers were fixed in situ with 2.5% glutaraldehyde in pH 7.4, 0.1 M cacodylate buffer for 1 hr at room temperature. The monolayer was postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr at room temperature. The monolayers were dehydrated and the flask was embedded in Epon 812. After polimerization the plastic was removed and ultrathin sections were cut parallel and perpendicular to the surface of the flask. The ultrathin sections were examined with uranyl

acetate-lead citrate staining in a Hitachi HS-8 electron microscope.

Enzyme histochemistry

The cortex-derived cells were subcultured into dual chamber tissue culture slides (Lab-Tek, Miles Laboratories, Naperville, Illinois) whose growth surface was enhanced with a bovine type I collagen coat followed by absorption of 1 ml of fetal calf serum proteins for 12 hr at 4°C. The cells were inoculated into the chamber slides at 33% confluency (a 1:3 subculture ratio) in the serum-free medium previously described. The cells were fed fresh growth medium every 3 days until 80 to 90% confluency at which time they were utilized for histochemistry. All histochemical determinations were performed in triplicate, with one chamber of each slide utilized as the control (incubating solution without substrate) while the enzyme determination was performed in the adjacent chamber. Additional controls included 5-μ sections of frozen kidney and cultured normal human skin fibroblasts identically subcultured into chamber slides. The growth medium utilized for the fibroblasts was DME containing 15% v/v fetal calf serum. The fibroblast cell strain, GM 0969, was obtained from the Human Genetic Mutant Cell Repository, Camden, New Jersey.

For the determination of acid phosphatase, alkaline phosphatase, β-glucuronidase, NAD diaphorase, NADP diaphorase, and succinic dehydrogenase, the chamber slides were rinsed three times with PBS and fixed for 30 sec in citrate-acetone fixative (40% 0.038 M citrate-citric acid buffer pH 5.4, 60% reagent grade acetone), rinsed well with distilled water, and allowed to air dry. Acid phosphatase was determined utilizing the naphthol AS-BI phosphate method [12, 13] and Sigma kit #386. For the demonstration of alkaline phosphatase the naphthol AS-MX method was used [14] and Sigma kit #85. β-Glucuronidase was determined with the naphthol AS-BI method [15]. The demonstration of the dehydrogenases (NAD diaphorase, NADP diaphorase, and succinic dehydrogenase) involved the reduction of nitro blue tetrazolium by the released hydrogen ions [16, 17].

Chamber slides to be stained for 5′-nucleotidase and nonspecific esterase were washed three times with PBS and fixed for 30 sec in citrate-acetone-methanol (36% 0.038 M citrate-citric acid buffer pH 5.4, 54% reagent grade acetone, and 10% reagent grade methanol), rinsed thoroughly with distilled water and allowed to air dry. The metal precipitation method of Wachstein and Meisel [18] was utilized to demonstrate 5′-nucleotidase. Nonspecific esterase was determined with the αnaphthyl acetate method of Gomori [19] and Sigma kit 90-A1.

The sensitivity of glucose-6-phosphatase to fixation required a different preparatory technique. Cells from a 75 cm^2 confluent flasks were rinsed three times with PBS, and centrifuged in a 50-ml centrifuge tube. The supernate was removed and the cells snap-frozen. Sections (5 μ) were stained for glucose-6-phosphatase with the lead method of Wachstein and Meisel [20]. Controls for this enzyme demonstration included an incubation medium of β-glycerophosphate replacing glucose-6-phosphate as substrate and the simultaneous demonstration of the enzyme in snap-frozen cultured hepatocytes provided by Dr. Maria Buse, Medical University of South Carolina. All histochemical stain results were judged independently by four investigators based on a minus to +3 scale with minus indicating no staining.

Table 1. Growth supplements tested as serum-free media components[a]

Supplement	Concentration range tested
Hydrocortisone	1 to 100 ng/ml
Triiodothyronine	1 to 15 pg/ml
Prostaglandin E_1	1 to 25 ng/ml
Prostaglandin $F_{2\alpha}$	1 to 25 ng/ml
Progesterone	2 to 20 ng/ml
Putrescine	0.1 to 1.0 mg/ml
Glucagon	50 to 250 ng/ml
Aldosterone	1 to 20 μg/ml
Testosterone	0.1 to 5 ng/ml
Dihydrotestosterone	0.1 to 5 ng/ml
Estradiol	1 to 5 ng/ml
Kallikrein	1 to 10 ng/ml
Calcium	0.4 to 4 μg/ml
L-Glutamine	15 to 1500 μg/ml
L-Cysteine	1 to 120 μg/ml
Fibroblast growth factor	1 to 25 ng/ml
Epidermal growth factor	10 to 100 ng/ml
Parathyroid hormone	1 to 10 ng/ml

[a] The growth medium supplements were obtained from Collaborative Research and prepared according to their product specifications. Human urinary kallikrein was a gift from Dr. R. Mayfield, Medical University of South Carolina, and was prepared in phosphate-buffered saline with 0.1% bovine serum albumin.

Immunohistochemistry

The avidin-biotin system was utilized for the immunohistochemical demonstration of keratin, Tamm-Horsfall glycoprotein, carbonic anhydrase, and factor VIII RAg [21] and Avidin-Biotin kit from Vector Labs, Burlingame, California. A 75 cm^2 confluent flask was fixed for 15 min with Carnoy's fixative, and rinsed three times with 70% ethanol. At this stage, the cells were rolled off the plastic with the aid of a rubber policeman and centrifuged. The cell pellet was routinely dehydrated and paraffin-embedded. Sections of cells, 6-μ thick, were deparaffinized and rehydrated prior to staining. In all cases the antiserum was at a 1:500 dilution. Controls included staining of serial sections with non-immune serum and staining of sections of Carnoy-fixed human kidney.

Results

Primary explant of cells

The ability to initiate a homogenous epithelial cell population from the cortex of the human kidney was attempted using a series of serum-free hormonally defined growth media formulations. The criterion utilized for judging the success of serum-free medium formulations was the explantation of a cell monolayer possessing an epithelial morphology of homogenous appearance free of cellular vacuolations at the light microscopic level. The basal serum-free medium used to assess growth factors was a 1:1 mixture of DME and Ham's F-12 (DME/F12) supplemented with selenium (5 ng/ml), insulin (5 μg/ml), and transferrin (5 μg/ml). To this basal medium the additions listed in Table 1 were tested by adding each component singly and monitoring the ability of cells to explant from the tissue fragment. The additions were tested at the lowest, highest, and middle concentration ranges. The cells were fed fresh growth media every 3 days. The results of this protocol demonstrated that hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml),

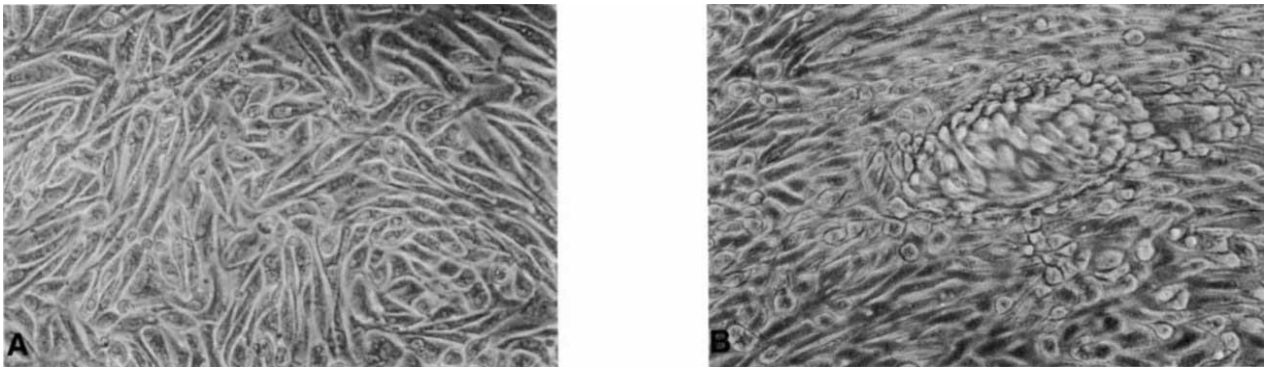

Fig. 1. *The morphology of epithelial cells explanted from the cortex of human kidney.* **A** *After 3 weeks in culture at which time a confluent monolayer has formed.* **B** *After an additional week in culture at which time hemicyst formation was apparent.* Photographs were taken with an Olympus 1 M inverted microscope. Growth conditions were as described in **Results.** The results shown are representative of ten independent explants with all displaying consistent results. (100×)

prostaglandin E_1 (10 ng/ml), and epidermal growth factor (10 ng/ml) are beneficial for epithelial cell monolayer formation; although in no instance was a confluent monolayer of homogeneous composition attained.

To further improve the possibility of obtaining a homogenous epithelial cell population, the above explant protocol was repeated by adding all possible combinations of the above four growth factors to the basal medium and monitoring explant formation. The results of this procedure demonstrated that one growth medium formulation gave rise to a fast-growing, morphologically homogenous, epithelial-like cell population. The growth medium formulation was the basal serum-free medium supplemented with hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and EGF (10 ng/ml). The cells proliferating from the explants became a confluent monolayer within 3 weeks at which time they were photographed using an inverted microscope (Fig. 1**A**). These cells, when maintained with continued feeding for an additional week after monolayer formation, formed hemicysts (domes) indicative of transporting epithelial cells (Fig. 1**B**).

Ultrastructural examination of the cells in a parallel plane of section from the cortical explant demonstrated polygonal cells with broad areas of juxtaposition between adjacent cells (Fig. 2). Microvilli (400 to 600 nm in length) projected into the intracellular spaces along these areas of juxtaposition with desmosomes interspersed. Frequent lumen formation was seen between cells. These structures were a general feature of these explanted cells and were usually formed with four to six cells around a central lumen. An examination of over 200 cell profiles from electron micrographs demonstrated that over 90% of the cells were arranged in this pattern. The cells forming these structures contained tight junctions (zonula occludens), intermediary junctions (zonula adherens), and desmosomes. The cells projected short fuzzy coated microvilli (100 nm in diameter and 300 to 400 nm in length) into the lumen. In general the cells examined had a rounded nucleus with dispersed chromatin, abundant free ribosomes, and scattered rough endoplasmic reticulum. The cytoplasm also contained a normal complement of mitochondria and lysosomes. Frequent microfilaments were seen which tended to encircle the nucleus.

Ultrastructural examination was also performed on cultures sectioned perpendicular to the growth surface. The results agreed with the above-described general features of the cells (photograph not shown). There were several additional features noted in this perpendicular plane of section. When viewed in this manner, the cultures were found to consist of a one cell thick monolayer and not a multilayer of cells. The interaction of the cell monolayer with the culture vessel surface was also assessed, and the formation of a basement membrane underlying the basal portion of the cells was not evident to an extent greater than that noted for the artificial collagen-fetal calf serum matrix already present on the growth surface (as noted by embedding treated flasks without cells). The frequent formation of a lumen by two adjacent cells with microvilli oriented intraluminally was also noted in the perpendicular plane of section indicating that the lumen formed by six to eight cells noted in the parallel plane of section was not an artifact. Sufficient serial sections could not be obtained to determine if these lumens were aligned into "tubule-like" structures.

Subculture of cortical cells

The confluent monolayers derived from cortical explants were subcultured by trypsin-EDTA (0.05%, 0.02%) and inoculated at a 1:3 subculture ratio into new flasks containing a bovine type 1 collagen surface and the serum-free growth medium described previously. The cells subcultured in this fashion displayed plating efficiencies of over 90% and exhibited doubling times of 24 to 48 hr and retainment of original morphology. These cells were able to be subcultured at a 1:3 ratio for four additional passages (passage 5, 15 to 20 cell generations) with retainment of original morphology and doubling time. At passage 6 to 7 cell growth became markedly reduced (doubling time of 72 to 96 hr) with a corresponding increase in cell size and loss of morphology. Within two additional passages the cells lost all proliferative capacity and eventually detached from the culture surface. The growth factors tested previously for explant formation (Table 1) were once again assessed to determine if additional components could increase culture life span. All were without affect on this parameter.

In an effort to further improve the in vitro life span of these cells, the extracellular matrix used to coat the culture vessel surface was assessed. The extracellular matrix materials assessed included: normal plastic growth surface, collagen (bo-

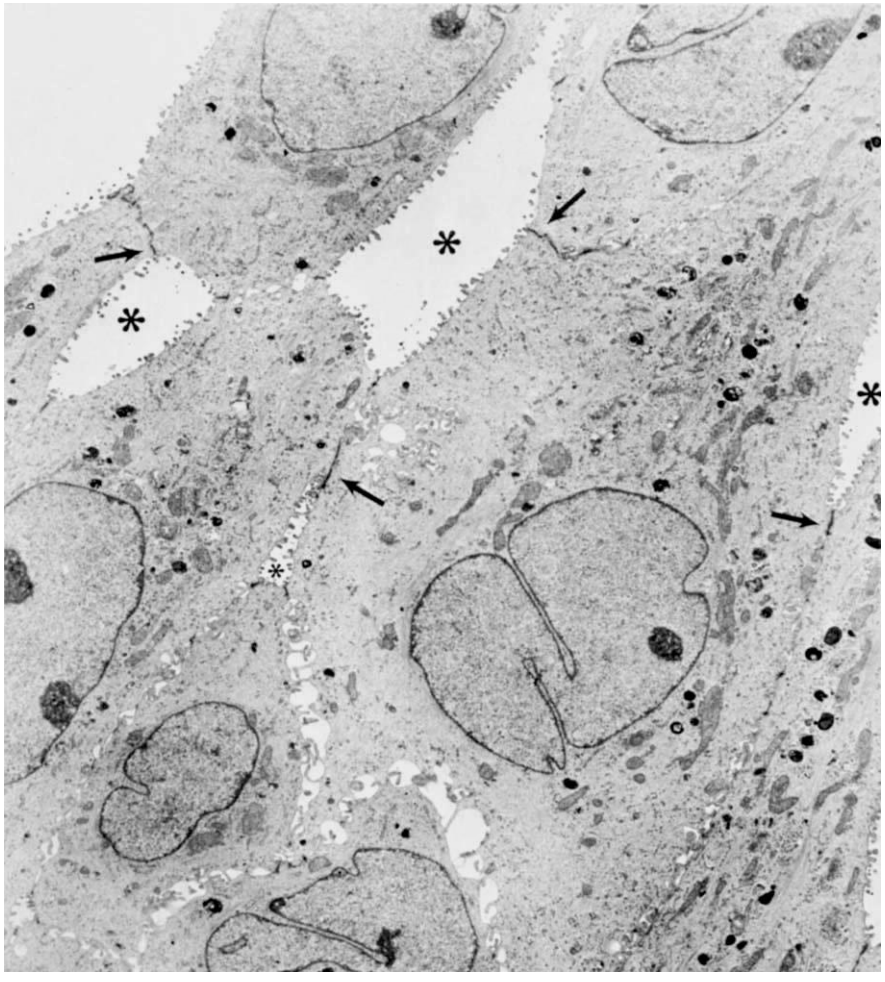

Fig. 2. *The ultrastructural characteristics of the epithelial cells depicted in Figure 1A.* The monolayers were fixed and processed for electron microscopy as described in **Methods.** Symbols are: *asterisks,* lumen; *arrows,* junctional complexes. (3,675×)

vine, type I), fibronectin (human), collagen followed by fibronectin, and a collagen coat followed by absorption of fetal calf serum components. The results of this study clearly demonstrated that a collagen matrix with absorbed fetal calf serum proteins yielded a significant increase in culture life span to 16 passages (Table 2). This increase in culture life span was not a result of increased growth rate since doubling times remained between 24 and 48 hr. The cells retained original morphology and ability to dome identical to that noted for the original explants (Fig. 1**A** and **B**). The cells between passage 17 to 22 began to lose proliferative capacity with an increase in cell size and failed to proliferate within one or two additional subcultures. Cells cryopreserved at passage 2, upon thawing, regained their original morphology, cell doubling times, and culture life span.

Characteristics of subcultured cells

The cortex-derived cells were characterized extensively between passage 6 to 12 to determine that they were indeed of a homogenous origin, nonfibroblastic in nature, and that they retained the characteristics of explant cultures. These cells exhibited morphology at the light microscopic level identical to that of the original explant culture (Fig. 1**A**) and retained their ability to form hemicysts if held at confluency for an additional week (Fig. 1**B**). Ultrastructural examination of these cells with transmission electron microscopy revealed only one change from the explant cultures; this was a small increase in the number of lysosomes and intracellular vacuoles. The luminal organization and cell-to-cell junctions were identical in nature to that described previously for explant cultures.

The nonfibroblast nature of these cells was strongly suggested by the finding of numerous areas of cell-to-cell attachment in ultrastructural examination; however, a low-level of fibroblast contamination could not be eliminated. Further evidence that fibroblasts were not present was obtained by assessing the serum-free medium for its ability to sustain the growth of a human diploid fibroblast cell strain (GM0969) and also for its ability to support the explantation of fibroblasts from a portion of human skin. The results of this study demonstrated that the human diploid fibroblast cell strain, subcultured into the serum-free growth medium, was unable to proliferate, and over 80% of the cells detached from the growth surface within 3 days. Continued feeding of the remaining attached cells over a 2-month period demonstrated that these cells could not adjust to this medium and regain proliferative capacity. Likewise, it was also demonstrated that skin specimens could not explant a fibroblast population when provided with serum-free growth medium over a 1-month period, nor when the serum-free

Table 2. Effect of extracellular matrix on culture life span[a]

Matrix	Passage number before senescence
Plastic	3
Fibronectin	3
Collagen	8
Collagen + Fibronectin	11
Collagen + Fetal calf serum proteins	16

[a] The extracellular matrix was prepared as described in **Methods**. The determinations were performed in triplicate from three different kidney specimens with consistent results.

medium was supplemented with conditioned medium from cultures of human kidney epithelial cells. In both cases fibroblasts were able to proliferate on DME growth medium containing 15% fetal calf serum.

Origin of the cortex-derived cells

To define the origin within the human nephron of the cortex-derived cells, a series of enzyme histochemical and immunohistochemical determinations were performed. The enzyme and immunohistochemical determinations performed as well as the reported distribution of these within the human nephron are displayed in Table 3. This reported distribution was confirmed by observing frozen sections of human kidney and in no instance did the reported distribution differ significantly from our independent observations. Identical determinations (Table 3) were performed on the cortex-derived cells and a human fibroblast cell strain. An inspection of the enzyme distribution for the cortex-derived cells clearly demonstrates these cells stain identically to those found in the proximal tubules of the human nephron in vivo. This clearly indicates that these cells are derived from this segment. Their origin from the proximal tubule is strongly reinforced by the presence of glucose-6-phosphatase activity in the cultured cells, as well as the finding of weak reactivity for carbonic anhydrase.

Discussion

The goal of the present study was to establish a human kidney epithelial cell strain of defined origin in cell culture to assess the factors underlying kidney diseases and disorders. To attain this goal several criteria must be considered and fulfilled. The cell strain developed must proliferate well in culture and be capable of extended subculture such that laboratories with limited culture facilities can routinely grow and maintain their own cultures. Serum-free hormonally defined culture medium used in place of serum-containing medium is also desirable since studies assessing, for example, prostaglandin metabolism, are severely hampered by the endogenous prostaglandins present in serum. The origin within the nephron of the cell strain isolated must be defined and, in addition, the cell strain must retain many in vivo properties if it is to serve as a model for in vivo kidney metabolism.

The results clearly indicate that the kidney epithelial cell strain isolated in this study can be serially propagated with an efficiency usually reserved only for fibroblast cells. The cell strain isolated can undergo extensive subculture to 16 passages at a 1:3 subculture ratio with retainment of original morphology and doubling time. The procedure for subculturing cells is very similar to that utilized for diploid human fibroblasts. The trypsin concentration was lowered to 0.05% from 0.25% but is commercially available. The trypsin action was inhibited by an equal volume of fetal calf serum since this is a routine procedure in fibroblast cell cultures. However, for studies where no exposure to serum is a requirement, a 0.1% solution of soybean trypsin inhibitor can be utilized without adverse effect on cell growth. Serum-free growth medium presents little problem since all components are commercially available as sterile powders. The matrix coating of the culture surface is likewise routine with easily followed directions from the supplier.

The in vitro life span of the cortex-derived cells can be conservatively estimated to be 30 to 35 generations if one assumes an estimate of 10 to 15 generations for the formation of a confluent monolayer from the initial explant. Attainment of a total of 30 to 35 generations is well within the range of that expected for normal cells in culture as defined by Hayflick for diploid human fibroblasts [8]. The proliferation through 16 passages at a 1:3 subculture ratio would allow the theoretical accumulation of over 10,000 75 cm^2 T-flasks by passage 11 from a single explant in a 25 cm^2 T-flask. This coupled with the fact that the cells can be preserved in liquid nitrogen and that one kidney can initiate over 100 explants clearly underscores the ease of use of the present culture system.

To define the site of origin of the cortex-derived cells as well as to judge the extent of retainment of in vivo properties, a series of enzyme histochemical and immunohistochemical staining techniques were employed. For histochemical markers to succeed in defining the site of origin within the nephron, the cells must retain most in vivo characteristics. This approach is in marked contrast to other identification methods. For example, the production of monoclonal antibodies to cultured cells eventuates in the formation of a multitude of monoclonal isolates recognizing many cell types, with only a small percentage specific for the cell in question [22].

The results of both the morphological and histochemical examination of these cells define the proximal tubule of the human nephron as the site of origin. The ultrastructural examination clearly demonstrated these cells to be epithelial in nature by the presence of numerous intercellular junctions. This was further reinforced by negative immunostaining for factor VIII RAg, an endothelial cell surface marker [23] and by an enzyme histochemical profile very distinct from that of a human fibroblast cell strain. In addition, the cortex-derived cells formed hemicysts, a characteristic of transporting epithelia in vitro [9, 24]. The remaining immunohistochemical determinations suggest where the cells did not originate from within the nephron. Negative keratin staining suggested an origin distinct from transitional epithelia and the collecting duct [25]; negative Tamm-Horsfall reactivity suggested an origin distinct from the distal convoluted tubule and the thick ascending limb of the loop of Henle [26]; and weak reactivity for carbonic anhydrase suggests the origin as proximal tubule [27]. The only characteristic of these cells not in agreement with an origin from the proximal tubule was the absence of a brush border. To our knowledge, no cell in culture has been demonstrated to retain a brush border nor have primary cultures of rabbit and pig proximal tubule cells grown by other investigators retained a brush border [10, 11].

The enzyme histochemical profiles further identified the site

Table 3. Histochemistry of the human nephron and cultured cells[a]

Demonstration of	Human nephron						Cultured cells	
	GL	PR	DES	ASC	DC	CT	Cortex	Fibro
Acid phosphatase	++	++	+	+	+	++	++	+++
Alkaline phosphatase	−	++	−	−	−	−	+	−
Glucose-6-phosphatase	−	++	−	−	−	−	++	±
β-Glucuronidase	−	+	−	+	+	+	+	+
Nonspecific esterase	±	+++	−	+	++	+	+++	+
5'Nucleotidase	++	+	−	−	−	−	++	±
NAD diaphorase	+	+++	++	++	++	+++	+++	+
NADP diaphorase	+	++	+	±	±	±	++	+
Succinic dehydrogenase	−	++	−	+++	+++	+	++	−
Keratin	−	−	−	−	+	++	−	−
Carbonic anhydrase	−	+	−	−	++	++	+	−
Factor VIII RAg	−	−	−	−	−	−	−	−
Tamm-Horsfall glycoprotein	−	−	−	++	++	+	−	−

Abbreviations utilized for localization within the human kidney are: GL, glomerulus; PR, proximal tubule; DES, descending limb of Henle's loop; ASC, ascending limb of Henle's loop; DC, distal convoluted tubules; CT, collecting tubules. Abbreviations utilized to designate the cultured cell strains are: Cortex, the proximal tubule cell strain explanted form the cortex of the human kidney; Fibro, the fibroblast cell strain GM0969; the conditions for growth of the cell strains were as described in the text; both cell strains were utilized at passage 6 to 12.

[a] The procedures for the enzyme histochemical and immunohistochemical determinations are described in detail in the **Methods** section. The determinations were performed in triplicate and for the epithelial cell strain isolates from three separate kidneys were tested. In no case was there a major judging difference between four independent observers of the determinations. Factor VIII was positive in the vessels of the kidneys examined.

of origin. While all the enzyme reactions were consistent for a proximal tubule origin, the positive reactivity for glucose-6-phosphatase was central for assigning origin from the proximal tubule. Further evidence was obtained to reinforce that indeed this reaction was specific for glucose-6-phosphatase since other phosphatases can yield false positive results [20, 28]. In addition, the enzyme histochemical demonstration of glucose-6-phosphatase is known to be extremely sensitive to fixation procedures whereas the other phosphatases are not [20, 28]. Glucose-6-phosphatase demonstration in both the cortex-derived epithelial cells and cultured hepatocytes in the present study was extremely fixation sensitive, with all reactivity abolished with even a 10-sec fixation. To obtain positive glucose-6-phosphatase reactivity in liver and cortex-derived cells, treatment in a fashion similar to that used for frozen tissue sections was necessary. The cells were snap-frozen without fixation and sectioned with a cryostat. Only under these conditions could a positive reaction for glucose-6-phosphatase be obtained. The sensitivity to fixation adds further conclusive evidence that glucose-6-phosphatase is present in the cortex-derived cells. Studies are underway to localize this enzyme at the ultrastructural level.

The purity of the proximal tubule cell strain was also assessed by the enzyme histochemical stains. In all instances the histochemical reactions were homogenous in intensity. Histochemical demonstrations were performed at a variety of passage numbers and from cultures explanted from different kidneys. All enzyme profiles were identical. The proximal kidney cell strain could possibly be heterogenous with respect to the proximal tubule although the weak reactivity for carbonic anhydrase suggests an origin from the convoluted segment of the proximal tubule [27]. The site of origin of the cortex-derived cells may be further elucidated by the production of monoclonal antibodies, a study presently underway in this laboratory.

The conditions necessary for growth and identification of human proximal tubule cells have been defined which allows research groups possessing minimal culture facilities to use this cell strain in studying diseases and disorders of the human kidney.

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